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Chromophore-bearing NH_2-terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability

(photomorphogenesis/regulatory photoreceptor/structure–function analysis/functional domains/specific degradation)

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ABSTRACT **In early seedling development, far-red-light-induced deetiolation is mediated primarily by phytochrome A (phyA), whereas red-light-induced deetiolation is mediated primarily by phytochrome B (phyB). To map the molecular determinants responsible for this photosensory specificity, we tested the activities of two reciprocal phyA/phyB chimeras in diagnostic light regimes using overexpression in transgenic *Arabidopsis*. Although previous data have shown that the NH_2-terminal halves of phyA and phyB each separately lack normal activity, fusion of the NH_2-terminal half of phyA to the COOH-terminal half of phyB (phyAB) and the reciprocal fusion (phyBA) resulted in biologically active phytochromes. The behavior of these two chimeras in red and far-red light indicates: (*i*) that the NH_2-terminal halves of phyA and phyB determine their respective photosensory specificities; (*ii*) that the COOH-terminal halves of the two photoreceptors are necessary for regulatory activity but are reciprocally interchangeable and thus carry functionally equivalent determinants; and (*iii*) that the NH_2-terminal halves of phyA and phyB carry determinants that direct the differential light lability of the two molecules. The present findings suggest that the contrasting photosensory information gathered by phyA and phyB through their NH_2-terminal halves may be transduced to downstream signaling components through a common biochemical mechanism involving the regulatory activity of the COOH-terminal domains of the photoreceptors.**

Higher plants have evolved several photosensory systems to measure light quality, quantity, periodicity, and direction. The best characterized of these photoreceptors are the red and far-red light-responsive phytochromes, which control such diverse responses as seed germination, deetiolation (inhibition of hypocotyl elongation, hook-opening, cotyledon separation and expansion), shade avoidance, and flowering (1, 2).

Phytochromes are dimers with apoprotein subunits of ≈125 kDa (1). Each subunit has two major structural domains (3): (*i*) a globular NH_2-terminal domain that carries the covalently bound chromophore (3) and (*ii*) an extended COOH-terminal domain that carries the dimerization sites (4, 5). After synthesis in the red-absorbing phytochrome (Pr) form, photoconversion to the active far-red-absorbing phytochrome (Pfr) form is required for most responses. The biochemical mechanism of phytochrome action is not understood.

In *Arabidopsis* the phytochrome gene family has five members: *PHYA* to *PHYE* (6). Current information is most advanced regarding the structure and function of phytochrome A (phyA) and phytochrome B (phyB) (7–11). phyA and phyB are 50% identical at the amino acid level (6, 12) and have similar spectral properties (7). phyA is the most abundant in dark-grown tissues (*ca.* 50× phyB levels). However, because of light-dependent phyA degradation, light-stable phyB becomes the more abundant in the light (*ca.* 2× phyA levels) (13).

Mutants in both loci have been defined in *Arabidopsis* (10, 11, 14). *phyA* null mutants do not deetiolate in continuous far-red light (FRc) but are indistinguishable from the wild-type (wt) in continuous red light (Rc) (10, 15). Conversely, *phyB* null mutants are defective in Rc-induced deetiolation but are wt in FRc (9). Thus phyA and phyB are necessary and primarily responsible for deetiolation in FRc and Rc, respectively. Furthermore, constitutive overexpression of phyB in transgenic *Arabidopsis* causes enhanced inhibition of hypocotyl elongation (enhanced deetiolation) in Rc but not in FRc (7, 9), whereas constitutive overexpression of phyA causes enhanced inhibition of hypocotyl elongation in both Rc and FRc (9, 16). Therefore, the photosensory specificity of overexpressed phyA and phyB can be most readily differentiated in FRc. Nevertheless, the specificities of the overexpressed molecules can also be distinguished in Rc, where phyA is approximately 1/50th as active as phyB on a per mole basis (7, 16). The available evidence indicates that differential photosensory specificity is most likely due to sequence differences between phyA and phyB (7, 9, 17). To localize the molecular determinants responsible for photosensory specificity, we have used overexpression of phyA/B chimeras in transgenic *Arabidopsis*.

MATERIALS AND METHODS

Construction of Chimeric Coding Sequences. Reciprocal fusions were created between the NH_2- and COOH-terminal domains of oat phyA and rice phyB at residue 615 and 659, respectively (phyAB and phyBA, Fig. 1). These two residues occupy the same position in the aligned sequences but are numbered differently because of an NH_2-terminal extension in phyB. To create the chimeras, an existing *Pst* I site in the rice phyB cDNA was deleted, and a new *Pst* I site was created by mutagenesis (18). This modification resulted in a Ser-659 → Gln change in the encoded protein (phyBM). phyAB and phyBA were created by using the new phyBM *Pst* I site and an existing, corresponding site in phyA.

Plant Transformation and Seedling Growth. *Arabidopsis* transformation and seedling growth were as described (16). Constitutive expression of all phytochromes was driven by the viral 35S promoter (19). At least 10 independent homozygous transformants were examined for each construct, all of which

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Abbreviations: phyA, phytochrome A; phyB, phytochrome B; Rc and FRc, continuous red and far-red light; wt, wild-type; mAb and pAb, monoclonal and polyclonal antibody; WLc, continuous white light.

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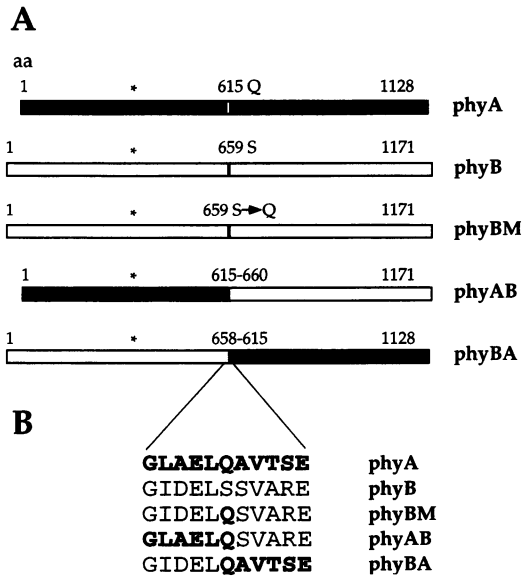

FIG. 1. (*A*) Schematic of aligned oat phyA (solid boxes) and rice phyB (open boxes) coding sequences used in this study. Amino acid residues are numbered above the sequences, and the amino acid residue at the fusion point is indicated. For phyBM Ser-659 of phyB was mutated to glutamine. For phyAB, residues 1–615 of phyA were fused to residues 660–1171 of phyB. For phyBA, residues 1–658 of phyB were fused to residues 615–1128 of phyA. *, Chromophore attachment site. (*B*) Alignments of the amino acid sequences near the fusion point of the phytochrome chimeras. The phyA sequence is in boldface letters, whereas the phyB sequence is in normal type. The single-letter amino acid code is used throughout.

showed qualitatively the same phenotype. For all constructs, the highest transgenic protein-expressing line obtained is shown, except for phyBM, which was chosen to match the phyB and phyBA expression levels, and for phyAB, where the highest expressing line (phyAB-1) and four additional, independent lines expressing phyAB at lower levels (phyAB-2 through phyAB-5) were included for comparison. For hypocotyl measurements, seedlings were essentially grown as described (16). The fluence rates used were: Rc, 0.017 μmol/m^2 per s and 22.0 μmol/m^2 per s; FRc, 1.3 μmol/m^2 per s and 5.0 μmol/m^2 per s; and continuous white light (WLc), 2.4 μmol/m^2 per s. Light sources and fluence rate measurements were as described (7).

Plant Extraction, Immunoblotting, and Spectrophotometric Measurements. Crude and ammonium sulfate-concentrated extracts, gel electrophoresis, and immunoblotting procedures were as described (7). Extracts from the same treatment were loaded on an equal, crude protein basis, whereas for comparisons of different light treatments with each other, extracts were loaded on a per gram fresh weight basis. $\Delta\Delta A$ measurements were performed as described (7).

Antibodies. The two monoclonal antibodies (mAbs) that recognize epitopes on the NH_2-terminal half of monocot (A1) and monocot and dicot (A2) phyA have been described (20). The mAb that recognizes an NH_2-terminal epitope of both monocot and dicot phyB (B1) has been described (7). A new mAb (B2) that recognizes the COOH-terminal domain of phyB from both monocots and dicots was identified from an antibody pool (8). The epitopes recognized by the phyB mAbs were localized by using plants overexpressing phyB deletions (D. Wagner, unpublished). Polyclonal phyB antiserum (pAb B3) was made monocot-specific as described (7).

RESULTS

Construction and Overexpression of Phytochrome Chimeras. To localize determinants for photosensory specificity, the two structural NH_2-terminal and COOH-terminal domains of oat phyA and rice phyB were fused at the corresponding residue in each sequence (amino acids 615 in oat phyA and 659 in rice phyB) to create reciprocal fusion proteins (phyAB and phyBA, Fig. 1*A*). The amino acid sequence at the fusion point for each construct is shown in Fig. 1*B*. The chimeras were created from two monocot phytochromes to facilitate immunochemical discrimination between the overexpressed (monocot) and endogenous (dicot) phyA and phyB with specific antibodies (7, 20). Plants overexpressing full-length oat phyA (21) and rice phyB (7) have been described previously.

Levels of phytochrome expression in the transformants were determined by using immunoblot and spectrophotometric assays. mAb A1 (monocot-phyA, NH_2-terminal specific) detects high levels of expression in the phyA and phyAB-1 lines (Fig. 2*A*). The phyAB chimera migrates faster than oat phyA [124 kDa (22)], closer to the 122-kDa molecular mass of rice phyB (7). mAb B1 (monocot/dicot-phyB, NH_2-terminal specific) detects equal levels of overexpression in phyBM-, phyB-, and phyBA-overexpressing seedlings (Fig. 2*B*). The lower intensity band in the phyA and phyAB-1 lines is equivalent to that in wt and represents endogenous phyB (Fig. 2*B*, lanes 4–6). mAb B2 (monocot/dicot-phyB,

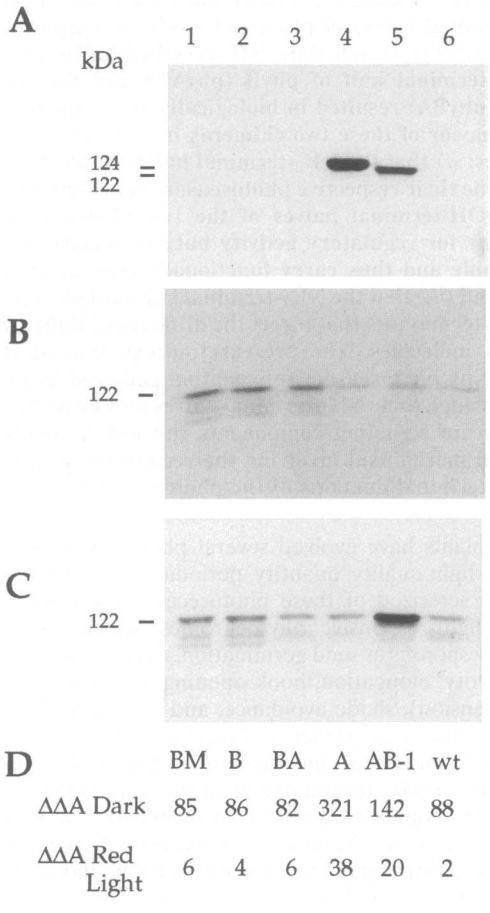

FIG. 2. (*A–C*) Immunoblot detection of phytochrome in crude extracts from 7-day-old dark-grown *Arabidopsis* seedlings. Twenty micrograms of crude protein was loaded per lane on all immunoblots. Extracts from seedlings overexpressing either phyBM (BM, lane 1), phyB (B, lane 2), phyBA (BA, lane 3), phyA (A, lane 4), phyAB (AB-1, lane 5), or wild-type (wt, lane 6) were probed with mAb A1 (monocot-phyA, NH_2-terminal specific) (*A*), mAb B1 (monocot/dicot-phyB, NH_2-terminal specific) (*B*), or mAb B2 (monocot/dicot-phyB, COOH-terminal specific) (*C*). (*D*) Spectrophotometric analysis of 10-fold concentrated ammonium sulfate-concentrated extracts from 7-day-old seedlings grown in the dark or in Rc. $\Delta\Delta A$ measurements represent the amount of spectrally active phytochrome in arbitrary units per μg of crude protein.

COOH-terminal specific) detects a stronger band in the phyAB-1 than in the phyBM and phyB lines, whereas extracts from phyBA and phyA transformants show only the fainter endogenous phyB band seen in wt extracts (Fig. 2*C*). Thus, proteins carrying the phyA NH_2-terminal domain are present at higher levels than those carrying the phyB NH_2-terminal domain, with the phyAB-1 line overexpressing the transgenic protein at about half the level of the phyA line. The amount of spectrally active phytochrome in dark grown seedlings of each line (Fig. 2*D*) parallels that on the immunoblot. Spectral activity of extracts from phyAB- and phyA-overexpressing seedlings is markedly higher than wt. In Rc-grown seedlings, spectral activity of phyBM, phyB, and phyBA is slightly higher than background (wt). Thus, all overexpressed phytochromes are spectrally active. The spectral activity increase of phyA and phyAB above wt is much reduced in Rc, suggesting that both overexpressed phyA and phyAB may be light labile.

NH_2-Terminal Domains Direct Photosensory Specificity in FRc and Rc. phyA has been shown to mediate the deetiolation response in FRc. Thus, the response of the chimeras to FRc serves as a diagnostic marker for phyA photosensory specificity. As shown in Fig. 3 *A* and *B*, three distinct phenotypes are exhibited in the transgenic lines in FRc. Seedlings carrying transgenes with the NH_2-terminal half of phyA (phyA or phyAB) display greater inhibition of hypocotyl elongation than wt in two different FRc fluence rates. In contrast, hypocotyl length in phyBM- and phyB-overexpressing seedlings is indistinguishable from wt in either FRc fluence rate. Strikingly, phyBA overexpression results in a decreased response in both FRc fluence rates as compared with wt or to phyB- and phyBM-overexpressors.

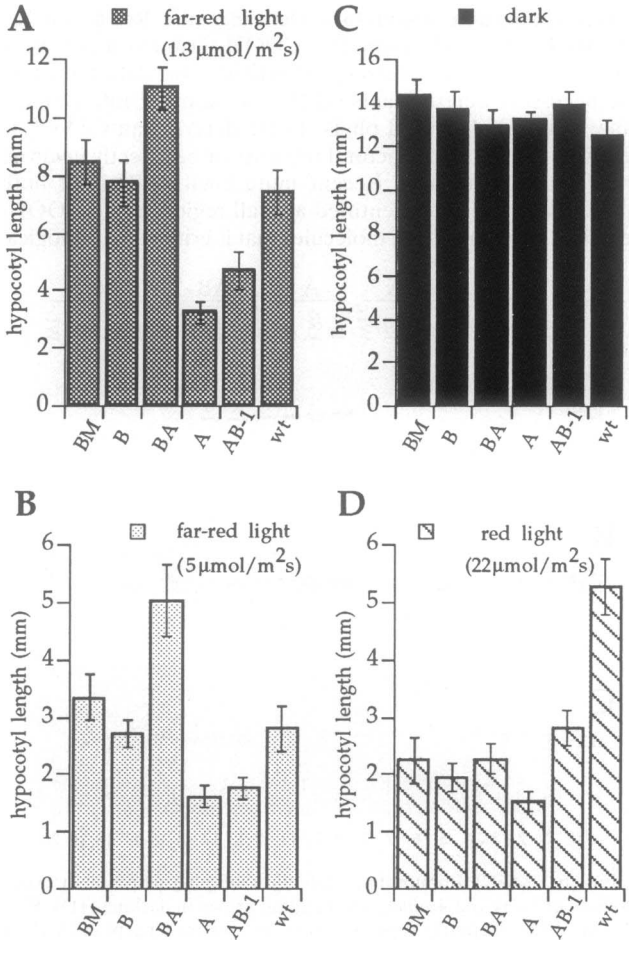

FIG. 3. Inhibition of hypocotyl elongation in 4-day-old wild-type (wt) and transgenic *Arabidopsis* seedlings overexpressing either phyBM (BM), phyB (B), phyBA (BA), phyA (A), or phyAB (AB-1). Seedlings were grown in low fluence rate FRc (*A*), higher fluence rate FRc (*B*), darkness (*C*), or Rc (*D*). Mean hypocotyl lengths were determined for more than 20 seedlings per line and light treatment. Error bars indicate 1 standard deviation. Note differences in scale between light treatments.

The somewhat lower responsiveness of phyAB-1- than phyA-transformants in lower fluence rate FRc may be accounted for by the lower levels of phyAB expression in this line (Fig. 2 *A* and *D*) (16). This deduction is supported by the finding that phyAB is as effective as phyA in higher fluence rate FRc (Fig. 3*B*), suggesting that phyAB is as active as phyA on a per mole basis in FRc. Thus, in the context of a full-length phytochrome molecule, the NH_2-terminal domain of phyA directs phyA photosensory specificity, whereas the COOH-terminal domain is neither exclusively necessary (as it is absent in the active phyAB construct) nor is it sufficient for the FRc response [as the phyBA chimera does not show phyA-like activity but instead causes a dominant negative (23) phenotype (Fig. 3*B*)]. The visible phenotypes of all lines in FRc are shown in Fig. 4. In the dark all the transgenic seedlings are indistinguishable from wt, indicating that the phenotypes observed are light-dependent (Fig. 3*C*).

Seedlings of all transgenic lines are significantly shorter than wt in Rc (Fig. 3*D*). Equal (low) levels of overexpressed protein in the phyBM, phyB, and phyBA lines (Fig. 2*B*) result in approximately equal inhibition of hypocotyl elongation in Rc, indicating that phyBM and phyBA are as active as phyB on a per mole basis. The high levels of phyA overexpression in the line used here result in even shorter seedlings, whereas seedlings overexpressing phyAB are taller than the phyA seedlings. This latter difference in height can be accounted for by the 50% lower level of overexpression in the phyAB-1 compared to the phyA line (Fig. 2 *A* and *D*) (16). Thus phyAB appears to be as active as phyA in Rc on a per mole basis. On the other hand, phyAB-1 seedlings are slightly taller than phyBM, phyB, and phyBA seedlings in this light condition despite the much higher level of overexpression in the phyAB-1 line (Fig. 2*C*), suggesting that phyAB may be less active than phyB on a per mole basis. However, these data do not exclude the possibility that the Rc deetiolation response is saturated in phyAB-1 and that much lower phyAB levels similar to those of the phyB line may already induce the full response. To investigate this possibility, four additional independent transgenic lines overexpressing phyAB at lower levels than phyAB-1 (Fig. 5*A*) were analyzed for responsiveness to low fluence rate Rc. The data show that inhibition of hypocotyl elongation does indeed decrease with decreasing phyAB levels such that phyAB-4 and phyAB-5, which have phyAB levels slightly higher than or equivalent to that of phyB in the phyB line, are similar to wt in responsiveness (Fig. 5*B*). Thus, like phyA (7, 16), but unlike phyBA, phyAB is less active in Rc than phyB on a per mole basis. The data indicate that, in the context of a full-length molecule, the NH_2-terminal domain of phyB is responsible for the higher efficiency of photon utilization by phyB than by phyA in Rc.

phyAB Is Light Labile. Potential for *in vivo*, light-dependent degradation of the overexpressed molecules was analyzed by using seedlings grown in darkness (D), FRc, Rc, and WLc (Fig. 6). Oat phyA levels were high in D or FRc and reduced in Rc and WLc (Fig. 6*A*) as previously described (16). Interestingly, the phyAB chimera behaved similarly. Extracts from all lines, including the dominant-negative phyBA overexpressors, show the same levels and degradation patterns of endogenous phyA, which migrates at 121 kDa (8), in response to the different light regimes, indicating that overexpression does not interfere with endogenous phyA expression or degradation (Fig. 6*B*). Fig. 6*C* shows that neither overexpressed phyBM nor phyBA were degraded in Rc or WLc in contrast to phyAB as measured with pAb B3 (monocot-phyB-specific, NH_2- and COOH-terminal reactive). The band intensities of phyBM, phyBA, and phyAB cannot be directly compared because phyBM carries both the

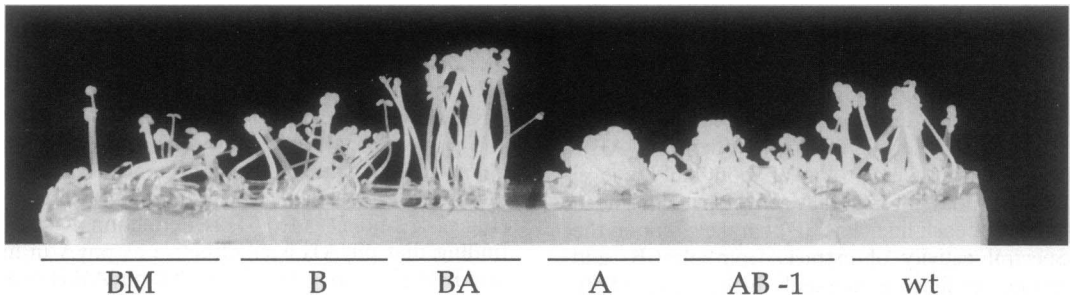

FIG. 4. Visible phenotypes of seedlings grown in FRc (1.3 μmol/m^2 per s) for 3 days. Seedlings represent lines overexpressing phyBM (BM), phyB (B), phyBA (BA), phyA (A), or phyAB (AB-1), and wt control.

NH_2- and COOH-terminal epitopes recognized by the antibody, whereas phyBA and phyAB each only carry a subset of these epitopes. It has been shown previously that overexpressed as well as endogenous phyB is not degraded in the light (7, 8). Three conclusions follow from these data: the determinants necessary to direct light-dependent phyA degradation reside on the phyA NH_2-terminal domain; the phyA COOH-terminal domain is not exclusively necessary for phyA-specific degradation; and the phyB COOH-terminal domain does not interfere with phyA-specific degradation.

DISCUSSION

Photosensory Specificity Resides on the NH_2-Terminal Domain. Both mutant and overexpression studies indicate that phyA and phyB are primarily responsible for seedling deetiolation in FRc and Rc, respectively. The NH_2-terminal domains of phyA (16) and phyB (D.W., unpublished data) are not biologically active when overexpressed alone, showing that the COOH-terminal domains are necessary for activity. The present data show that the NH_2-terminal half of phyA is necessary and sufficient to direct FRc photosensory specificity in the context of a full-length molecule. Conversely, the phyB NH_2-terminal domain is necessary and sufficient to direct Rc photosensory specificity in the context of a full-length molecule. Overexpression of a phyA deletion mutant has previously pointed to a possible involvement of a small NH_2-terminal domain region in phyA photosensory specificity: deletion of amino acid residues 3–52 causes loss of activity in FRc, yet the molecule is active in Rc (16). In addition the same small NH_2-terminal deletion of phyB (which also deletes the phyB-specific NH_2-terminal extension) causes a drastic reduction in phyB activity in Rc (D.W., unpublished data).

Interchangeable Regulatory Determinants Reside on the COOH-Terminal Domain. The COOH-terminal domains of phyA and phyB are necessary for activity. Our data show that the necessary determinants on this domain are interchangeable between phyA and phyB. These determinants could be required for general structural integrity or be directly involved in regulatory function. Recent mutational analysis of both phyA and phyB have identified a small region in the COOH-terminal domain of both molecules that is critical for biological

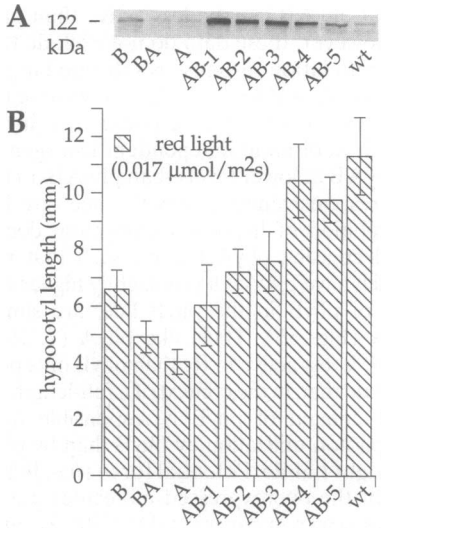

FIG. 5. Inhibition of hypocotyl elongation in Rc is dependent on phyAB protein levels. (*A*) Immunoblot analysis of phyAB levels in five independent transgenic lines (phyAB-1 through phyAB-5) overexpressing the chimeric phyAB protein, compared to phyB levels in the untransformed wild-type (wt) line and in lines overexpressing phyB (B), phyBA (BA), and phyA (A). Twenty micrograms of crude protein from extracts of 7-day-old dark-grown seedlings were loaded per lane, and the blot was probed with monoclonal antibody mAb B2, which detects an epitope on the COOH-terminal domain of monocot and dicot phyB. (*B*) Inhibition of hypocotyl elongation in Rc in 4-day-old seedlings overexpressing phyB (B), phyBA (BA), phyA (A), phyAB (AB-1 through AB-5) and wt. Mean hypocotyl lengths were determined for more than 30 seedlings. Each error bar denotes 1 standard deviation.

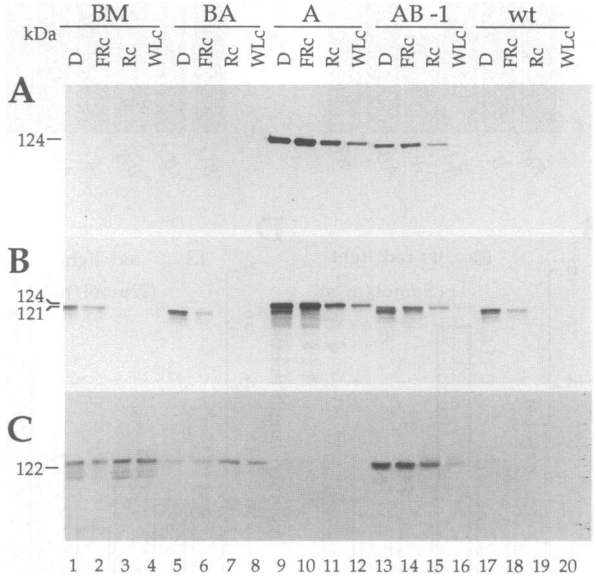

FIG. 6. (*A–C*) Immunoblot detection of phytochrome in crude extracts of 7-day-old *Arabidopsis* seedlings grown in darkness (D), FRc, Rc, or WLc. Seedlings represent lines overexpressing phyBM (BM, lanes 1–4), phyBA (BA, lanes 5–8), phyA (A, lanes 9–12), or phyAB (AB-1, lanes 13–16), and wt (lanes 17–20). Extract derived from 15 mg fresh weight was loaded per lane except for lanes 5–8 (phyBA) and 17–20 (wt) in *C*, which contain twice as much extract per lane. Crude protein concentrations were 18 (D), 33 (FRc), 78 (Rc), and 50 (WLc) μg per lane, except as noted for *C*. Immunoblots were probed with mAb A1 (monocot-phyA, NH_2-terminal specific) (*A*), mAb A2 (monocot/dicot-phyA, NH_2-terminal specific) (*B*), or pAb B3 (monocot-phyB specific, NH_2- and COOH-terminal reactive) (*C*).

but not spectral activity (10, 24–26). The data suggest that the COOH-terminal domains of both molecules have a direct role in regulatory function rather than simply a structural role. In addition, these and the current data suggest that the biochemical basis for the regulatory activity may be similar for phyA and phyB. This model predicts at least some conservation in the molecular determinants of the reaction partner(s) of the phyA and phyB signal transduction pathways. We propose that the specific activation by different light qualities of the NH_2-terminal domains of phyA and phyB is transduced to their COOH-terminal domains where functionally equivalent elements then interact with one or more downstream signaling components (Fig. 7).

Overexpression of phyBA reduces deetiolation in FRc compared to wt. This dominant negative interference with endogenous phyA activity is neither due to cosuppression [phyA expression and degradation are unaltered as compared to wt (Fig. 6)] nor to chromophore sequestration (spectral activity of endogenous phyA is the same in wt and phyBA-overexpressing plants [Fig. 2*D*, Dark $\Delta\Delta A$)]. Interference could be direct, by formation of inactive phyA/phyBA dimers, or indirect, by nonproductive interaction with a phyA signal transduction pathway component. Consistent with the former hypothesis, the COOH-terminal half of phyA contains the dimerization sites (5, 16, 27).

The NH_2-Terminal Domain of phyA Directs Light-Induced Targeted Degradation. phyA is specifically degraded in the light (16, 28), whereas phyB is light-stable (7, 8). Our data show that light-dependent degradation of phyA is determined by the NH_2-terminal domain of phyA. One possible signal motif for specific degradation of the Pfr form of phyA may be a PEST sequence (29), which is found only in phyA and is localized on the NH_2-terminal half of the molecule near the chromophore attachment site. However, because the NH_2-terminal domain of oat phyA is not degraded when expressed alone (16), determinants on the COOH-terminal domain appear to be necessary for degradation. The present data indicate that the phyB COOH-terminal domain can substitute for the phyA COOH-terminal domain in this respect. A ubiquitin-mediated mechanism of PfrA degradation has also been postulated (30). The putative ubiquitin attachment sites reside on the COOH-terminal domain and are conserved between phyA and phyB (31). The requirement for the presence of a COOH-terminal domain for degradation and the ability of phyB to substitute for this activity may be the result of this conserved attachment region. The phyB NH_2-terminal domain is sufficient to confer phyB-like degradation behavior to the phyA COOH-terminal domain. Recently, a NH_2-terminal signal for degradation with dependence on a COOH-terminal ubiquitination site was described for c-Jun (32). To identify the proteolytic signal for phyA, fusion of phyA subregions to unrelated proteins and analysis of specific degradation of the fusion protein would be most informative (32).

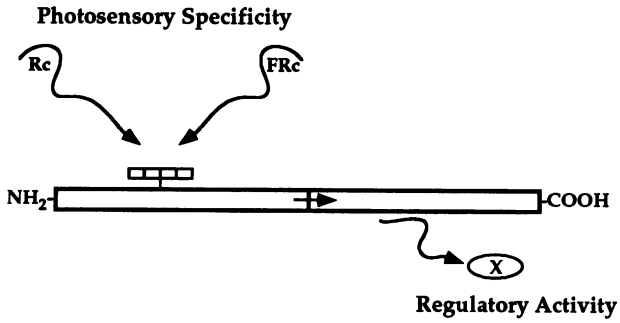

FIG. 7. Model of phytochrome mode of action. Determinants for phyA and phyB photosensory specificity toward FRc and Rc reside on the NH_2-terminal domain. Chromophore is indicated by four small open boxes. It is proposed that the signal for activation is communicated to the COOH-terminal domain, where interchangeable determinants of phyA or phyB are necessary for interaction with the reaction partner(s) (X) in the signal transduction pathway.

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